Example 4

Multifactorial optimization of the refolding of Interleukin-4 derivative employing the TRIS-sulfuric acid based system

An attractive combination of aggregation suppressors is the TRIS-base/H_2SO_4-system. Therefore, this system was chosen for further optimization employing a multifactorial analysis.

The total final volume of the refolding solution was 50 mL (glass vials, Schott, Germany). The glass vials were capped with parafilm. Refolding was allowed to run to completion within 24-36 hours with stirring on a magnetic bar stirrer (100-200 rpm). At intervals, samples were withdrawn and analyzed by RP-HPLC (see Example 1).

The protein solution from Example 2, containing denatured, sulfitolyzed protein, is diluted into refolding buffer to give a final protein concentration indicated in Table 3. The following aspects of refolding buffer composition were investigated: concentration of TRIS-base (0.5 to 3 [M]), H_2SO_4 (depending on TRIS-concentration; 0.4 to 1.4 [M]), residual guanidinium hydrochloride concentration (80-400 mM), L-cysteine concentration (0.4 to 4 [mM]), and initial protein concentration (50 to 1000 [mg/L]). The pH of the refolding buffer was adjusted to 7.5. All refolding mixtures contained 1 mM EDTA.

The experiments described in this example was designed to allow multifactorial statistical analysis of correctly folded Interleukin-4 derivative yield data in order to assess the importance of all single factors and all two-factor interactions. A partial cubic experimental design was generated and the resulting data were also analyzed employing a partial cubic model. The coefficients of the polynoms of the partial cubic model are given in Table 2.

Term	TRIS	CYS	Protein	Term	
0	0	0	0	CONSTANT	Linear terms
1	1	0	0	TRIS-H2SO4_[M]	
2	0	1	0	Cysteine_[mM]	
3	0	0	1	Protein_[mg/L]	
4	1	1	0	TRIS-H2SO4_[M]*Cysteine_[mM]	Interaction terms
5	1	0	1	TRIS-H2SO4_[M]*Protein_[mg/L]	
6	0	1	1	Cysteine_[mM]*Protein_[mg/L]	
7	2	0	0	TRIS-H2SO4_[M]^2	Quadratic terms
8	0	2	0	Cysteine_[mM]^2	
9	0	0	2	Protein_[mg/L]^2	
10	1	2	0	TRIS-H2SO4_[M]*Cysteine_[mM]^2	Partialcubic terms
11	2	1	0	TRIS-H2SO4_[M]^2*Cysteine_[mM]	
12	1	0	2	TRIS-H2SO4_[M]*Protein_[mg/L]^2	
13	2	0	1	TRIS-H2SO4_[M]^2*Protein_[mg/L]	
14	0	1	2	Cysteine_[mM]*Protein_[mg/L]^2	
15	0	2	1	Cysteine_[mM]^2*Protein_[mg/L]	

Table 2 Partial cubic model employed for the experimental design of the refolding optimization of Interleukin-4 R121D Y124D

Trial #	TRIS-base	Cysteine	Protein	Ref. Yield	Protein recovery	Overall refolding yield	Purity
				[mg/L]	[%]	[%]	[%]
1	3	4	50	9	81.3	18.00	22.1
2	3	0.4	1000	2.92	10.65	0.29	2.7
3	0.5	4	525	90.45	48.55	17.23	35.5
4	0.5	2.2	1000	137.7	46.34	13.77	29.7
5	1.75	4	1000	199.72	54.77	19.97	36.5
6	1.75	0.4	1000	25.05	27.23	2.50	9.2
7	3	2.2	50	11.3	60.79	22.60	37.2

Trial #	TRIS-base	Cysteine	Protein	Ref. Yield	Protein recovery	Overall refolding yield	Purity
8	0.5	4	50	10.37	53.54	20.73	38.7
9	3	0.4	525	68.32	55.36	13.01	23.5
10	0.5	0.4	525	81.82	36.66	15.58	42.5
11	1.75	0.4	50	13.6	62.08	27.21	43.8
12	0.5	0.4	1000	15.8	18.89	1.58	8.4
13	3	4	1000	176.31	43.94	17.63	40.1
14	1.75	4	525	131.19	68.94	24.99	36.2
15	1.3333	1.6	366.667	93.63	68.21	25.54	37.4
16	2.1667	1.6	366.667	91.95	69.77	25.08	35.9
17	3	2.8	683.333	134.38	55.72	19.67	35.3
18	0.5	1.6	683.333	110.68	48.94	16.20	33.1
20	2.1667	2.8	50	12.49	71.59	24.98	34.9
1	3	4	50	9.38	52.32	18.76	35.9
2	3	0.4	1000	8.94	18.32	0.89	4.9
3	0.5	4	525	97.16	50.43	18.51	36.7
4	0.5	2.2	1000	140.29	45.63	14.03	30.7
5	1.75	4	1000	197.53	56.85	19.75	34.7
6	1.75	0.4	1000	19.75	27.61	1.97	7.2
7	3	2.2	50	15.08	72.5	30.15	41.6

Table 3 Effect of solution conditions (TRIS-H_2SO_4-system) on Interleukin-4 R121D Y124D refolding yield, recovery of soluble protein, overall refolding yield and purity

The yields obtained with selected combinations of these components are shown in Table 3. Inspection of these results shows that, under the experimental conditions employed, the following trends were apparent: (1) best refolding yields are obtained at high protein concentrations (750-1000 [mg/L]); (2) best overall refolding yields are obtained at 250 to 650 [mg/L] total protein concentration; (3) the optimal L-cysteine concentration range is 2.5 to 4 [mM]; (4) the optimal Tris-H_2SO_4-

concentration range is 1.4 to 2.4 [M]; (5) best protein recovery is obtained at low protein concentrations (50 to 250 [mg/L]), high Tris-H_2SO_4-concentrations (2-3 [M]) and 2 to 3.5 [mM] L-cysteine; (6) best purity is obtained at high protein concentrations (400-1000 [mg/L]), high L-cysteine concentrations (2.5-4 [mM]). The purity is indepening on the Tris-H_2SO_4 concentration.

A compromise between optimal refolding yield, purity and protein recovery was identified employing the following settings: 500 mg/L total protein, 3.3 mM L-cysteine, 2 M Tris-H_2SO_4 and 1 mM EDTA.

Checkpoints employing these optimal conditions revealed that the predicted and measured response values fit reasonably well, indicating that the model is adequate.

Overall refolding yield	Predicted:	24.9 [%] (±1.84 StdErr)
	Measured:	25.4 [%] (±0.37 StdErr)
Protein recovery	Predicted:	65.9 [%] (±6.55 StdErr)
	Measured:	62.9 [%] (±0.63 StdErr)
Purity	Predicted:	38.6 [%] (±3.63 StdErr)
	Measured:	40.4 [%] (±0.45 StdErr)
Refolding yield	Predicted:	127 [mg/L] (±14.5 StdErr)
	Measured:	126.9 [mg/L] (±1.85 StdErr)

Example 5

Multifactorial optimization of the refolding of Interleukin-4 derivative employing the Triethanolamine-sulfuric acid based system

Another attractive combination of aggregation suppressors is the Triethanolamine (TEA) /H_2SO_4-system. Therefore, this system was chosen for further optimization and scale-up of the protein concentration.

The total final volume of the refolding solution was 50 mL (glass vials, Schott, Germany). The glass vials were capped with parafilm. Refolding was allowed to run to completion within 24-36 hours with stirring on a magnetic bar stirrer (100-200 rpm). At intervals, samples were withdrawn and analyzed by RP-HPLC (see Example 1).

The protein solution from Example 2, containing denatured, sulfitolyzed protein, is diluted into refolding buffer to give a final protein concentration indicated in Table 5. The following aspects of refolding buffer composition were investigated: concentration of TEA (1 to 2 [M]), H_2SO_4 (depending on TEA-concentration), residual guanidinium hydrochloride concentration (80-400 mM), L-cysteine concentration (0.4 to 10 [mM]), and initial protein concentration (50 to 1000 [mg/L]). The pH of the refolding buffer was adjusted to 7.5. All refolding mixtures contained 1 mM EDTA.

The experiments described in this example was designed to allow multifactorial statistical analysis of correctly folded Interleukin-4 derivative yield data in order to assess the importance of all single factors and all two-factor interactions. A partial cubic experimental design was generated and the resulting data were also analyzed employing a partial cubic model. The coefficients of the polynoms of the partial cubic model are given in Table 4.

Term	TEA	CYS	Protein	Term	
0	0	0	0	CONSTANT	
1	1	0	0	TEA-H2SO4_[M]	Linear terms
2	0	1	0	Cysteine_[mM]	
3	0	0	1	Protein_[mg/L]	
4	1	1	0	TEA-H2SO4_[M]*Cysteine_[mM]	Interaction terms
5	1	0	1	TEA-H2SO4_[M]*Protein_[mg/L]	
6	0	1	1	Cysteine_[mM]*Protein_[mg/L]	
7	2	0	0	TEA-H2SO4_[M]^2	Quadratic terms
8	0	2	0	Cysteine_[mM]^2	
9	0	0	2	Protein_[mg/L]^2	
10	1	2	0	TEA-H2SO4_[M]*Cysteine_[mM]^2	Partial cubic terms
11	2	1	0	TEA-H2SO4_[M]^2*Cysteine_[mM]	
12	1	0	2	TEA-H2SO4_[M]*Protein_[mg/L]^2	
13	2	0	1	TEA-H2SO4_[M]^2*Protein_[mg/L]	
14	0	1	2	Cysteine_[mM]*Protein_[mg/L]^2	
15	0	2	1	Cysteine_[mM]^2*Protein_[mg/L]	

Table 4 Partial cubic model employed for the experimental design of the refolding optimization of Interleukin-4 R121D Y124D

Trial #	TEA	Cysteine	Protein	Ref. Yield	Protein recovery	Overall refolding yield	Purity
[-]	[M]	[mM]	[mg/L]	[mg/L]	[%]	[%]	[%]
1	2	10	0.1	5.91	138.09	5.9	4.3
2	2	0.4	1	135.12	49.49	13.5	27.3
3	0.5	10	0.55	15.23	16.92	2.8	16.4
4	0.5	5.2	1	70.06	19.28	7	36.3
5	1.25	10	1	49.37	18.06	4.9	27.3
6	0.5	5.2	0.1	9.64	66.78	9.6	14.4
7	1.25	0.4	1	167.96	42.5	16.8	39.5
8	2	5.2	0.1	13.66	103.17	13.7	13.2
9	0.5	10	0.1	3.56	76.7	3.6	4.6

Trial #	TEA	Cysteine	Protein	Ref. Yield	Protein recovery	Overall refolding yield	Purity
10	2	0.4	0.55	45.39	54.66	8.3	15.1
11	0.5	0.4	0.55	68.41	31.3	12.4	39.7
12	1.25	0.4	0.1	30.09	77.34	30.1	38.9
13	0.5	0.4	1	90.11	21.55	9	41.8
14	2	10	1	63.75	22.94	6.4	27.8
15	0.5	0.4	0.1	24.18	59.26	24.2	40.8
16	1.25	10	0.55	43.47	31.42	7.9	25.2
17	1	3.6	0.4	107.54	61.7	26.9	43.6
18	1.5	3.6	0.4	118.95	70.26	29.7	42.3
19	2	6.8	0.7	115.96	45.83	16.6	36.1
20	0.5	3.6	0.7	97.81	32.69	14	42.7
21	1.5	6.8	0.1	12.29	75.29	12.3	16.3
1	2	10	0.1	6.51	91.62	6.5	7.1
2	2	0.4	1	136.71	44.08	13.7	31.0
3	0.5	10	0.55	17.13	13.98	3.1	22.3
4	0.5	5.2	1	68.29	17.34	6.8	39.4
5	1.25	10	1	53.25	16.96	5.3	31.4
6	0.5	5.2	0.1	10.75	44.69	10.8	24.1
7	1.25	0.4	1	170.11	39.82	17	42.7
8	2	5.2	0.1	18.01	81.64	18	22.1
9	0.5	10	0.1	4.92	43.65	4.9	11.3

Table 5 Effect of solution conditions (TEA-H_2SO_4-system) on Interleukin-4 R121D Y124D refolding yield, recovery of soluble protein, overall refolding yield and purity

The yields obtained with selected combination of these components are shown in Table 5. Inspection of these results shows that, under the experimental conditions employed, the following trends were apparent: (1) best refolding yields are obtained at high protein concentrations (750-1000 [mg/L]); (2) best overall refolding yields are obtained at 100 to 550 [mg/L] total protein concentration; (3) the optimal L-

cysteine concentration range is 0.4 to 4 [mM]; (4) the optimal TEA-H_2SO_4-concentration range is 1 to 1.6 [M]; (5) best protein recovery is obtained at low protein concentrations (50 to 250 [mg/L]), high TEA-H_2SO_4-concentrations (1.5-2 [M]) and 4 to 10 [mM] L-cysteine; (6) best purity is obtained at high protein concentrations (600-1000 [mg/L]), L-cysteine concentrations ranging between 0.4 and 4 [mM]) and at the TEA-H_2SO_4 concentrations ranging between 0.8 and 1.5 [M]. A compromise between optimal refolding yield, purity and protein recovery was identified employing the following settings: 500 mg/L total protein, 0.8 mM L-cysteine, 1.4 M TEA-H_2SO_4 and 1 mM EDTA.

Checkpoints employing these optimal conditions revealed that the predicted and measured response values fit reasonably well, indicating that the model is adequate.

Overall refolding yield	Predicted:	24.6 [%] (±4.1 StdErr)
	Measured:	24.3 [%] (±0.8 StdErr)
Protein recovery	Predicted:	52.8 [%] (±10.5 StdErr)
	Measured:	58.2 [%] (±4.5 StdErr)
Purity	Predicted:	43.2 [%] (±5.3 StdErr)
	Measured:	41.8 [%] (±3.9 StdErr)
Refolding yield	Predicted:	106.8 [mg/L] (±16.9 StdErr)
	Measured:	121.6 [mg/L] (±2.0 StdErr)

Example 6

Refolding of bovine pancreatic trypsin inhibitor (BPTI, aprotinin) employing the TRIS-sulfuric acid based system

In order to demonstrate that the TRIS/H_2SO_4-system can also be employed for the refolding of other proteins than Interleukin-4 derivatives, the TRIS/H_2SO_4-system was also optimized for BPTI.

The total final volume of the refolding solution was 50 mL (glass vials, Schott, Germany). The glass vials were capped with parafilm. Refolding was allowed to run to completion within 24-36 hours with stirring on a magnetic bar stirrer (100-200 rpm). At intervals, samples were withdrawn and analyzed by RP-HPLC (see Example 1).

The protein solution from Example 2, containing denatured, sulfitolyzed protein, is diluted into refolding buffer to give a final protein concentration indicated in Table 7. The following aspects of refolding buffer composition were investigated: concentration of TRIS (0 to 2 [M]), H_2SO_4 (depending on the concentration of TRIS-base), residual guanidinium hydrochloride concentration (80-400 mM), L-cysteine concentration (0.1 to 4 [mM]), and initial protein concentration (50 to 1000 [mg/L]). The pH of the refolding buffer was adjusted to 7.5. All refolding mixtures contained 1 mM EDTA.

The experiments described in this example was designed to allow multifactorial statistical analysis of correctly folded BPTI yield data in order to assess the importance of all single factors and all two-factor interactions. A partial cubic experimental design was generated and the resulting data were also analyzed employing a partial cubic model. The coefficients of the polynoms of the partial cubic model are given in Table 6.

Term	TRIS	CYS	Protein	Term	
0	0	0	0	CONSTANT	
1	1	0	0	TRIS-H2SO4_[M]	Linear terms
2	0	1	0	Cysteine_[mM]	
3	0	0	1	Protein_[mg/L]	
4	1	1	0	TRIS-H2SO4_[M]*Cysteine_[mM]	Interaction terms
5	1	0	1	TRIS-H2SO4_[M]*Protein_[mg/L]	
6	0	1	1	Cysteine_[mM]*Protein_[mg/L]	
7	2	0	0	TRIS-H2SO4_[M]^2	Quadratic terms
8	0	2	0	Cysteine_[mM]^2	
9	0	0	2	Protein_[mg/L]^2	
10	1	2	0	TRIS-H2SO4_[M]*Cysteine_[mM]^2	Partial cubic terms
11	2	1	0	TRIS-H2SO4_[M]^2*Cysteine_[mM]	
12	1	0	2	TRIS-H2SO4_[M]*Protein_[mg/L]^2	
13	2	0	1	TRIS-H2SO4_[M]^2*Protein_[mg/L]	
14	0	1	2	Cysteine_[mM]*Protein_[mg/L]^2	
15	0	2	1	Cysteine_[mM]^2*Protein_[mg/L]	

Table 6 Partial cubic model employed for the experimental design of the refolding optimization of BPTI

Trial #	TRIS-base	Cysteine	Protein	Ref. Yield	Protein recovery	Overall refolding yield	Purity
[-]	[M]	[mM]	[mg/L]	[mg/L]	[%]	[%]	[%]
1	2	4	50	20.43	85.69	40.86	47.7
2	2	0.1	1000	0	0	0	0
3	0	4	525	159.29	61.05	30.34095	49.7
4	0	2.05	1000	275.45	70.84	27.545	38.9
5	1	4	1000	256.9	71.75	25.69	35.8
6	0	2.05	50	35.67	136.48	71.34	52.3
7	1	0.1	1000	0	2.59	0	0
8	2	2.05	50	19.45	80.85	38.9	48.1
9	0	4	50	8.51	39.92	17.02	42.6
10	2	0.1	525	0	0	0	0
11	0	0.1	525	0	0.4	0	0
12	1	0.1	50	15.71	69.04	31.42	45.5

Trial #	TRIS-base	Cysteine	Protein	Ref. Yield	Protein recovery	Overall refolding yield	Purity
13	0	0.1	1000	0	1.05	0	0
14	2	4	1000	158.93	36.23	15.893	43.9
15	0	0.1	50	5.03	14.13	10.06	71.2
16	1	4	525	18.64	89.48	3.550476	4
17	0.6667	1.4	366.667	107.29	83.34	29.26088	35.1
18	1.3333	1.4	366.667	104.5	82.6	28.49997	34.5
19	2	2.7	683.333	97.4	41.66	14.25367	34.2
20	0	1.4	683.333	149.44	51.46	21.86928	42.5
21	1.3333	2.7	50	14.08	76.21	28.16	36.9
1	2	4	50	16.15	69.37	32.3	46.5
2	2	0.1	1000	1.47	3.44	0.147	4.3
3	0	4	525	162.49	58.91	30.95048	52.5
4	0	2.05	1000	273.77	68.91	27.377	39.7
5	1	4	1000	265.9	78.2	26.59	34
6	0	2.05	50	9.73	39.56	19.46	49.2
7	1	0.1	1000	0	0.94	0	0
8	2	2.05	50	19.18	77.88	38.36	49.3

Table 7 Effect of solution conditions on BPTI refolding yield, recovery of soluble protein and overall refolding yield

The yields obtained with selected combination of these components are shown in Table 7. Inspection of these results shows that, under the experimental conditions employed, the following trends were apparent: (1) best refolding yields are obtained at high protein concentrations (750-1000 [mg/L]); (2) best overall refolding yields are obtained at 500 to 1000 [mg/L] total protein concentration; (3) the optimal L-cysteine concentration range is 2.5 to 4 [mM]; (4) the optimal TRIS-H_2SO_4-concentration range is 0.2 to 1.0 [M]; (5) best protein recovery is obtained at low protein concentrations (50 to 100 [mg/L]), moderate TRIS-H_2SO_4-concentrations

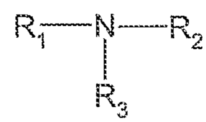
(0.9-1.4[M]) and 1.8 to 3.3 [mM] L-cysteine; (6) best purity is obtained at low protein concentrations (50-100 [mg/L]), L-cysteine concentrations ranging between 0.1 and 0.4 [mM]) and at the TRIS-H_2SO_4 concentrations ranging between 0.1 and 0.5 [M].

A compromise between optimal refolding yield, purity and protein recovery was identified employing the following settings: 700 mg/L total protein, 3.3 mM L-cysteine, 0.3 M TRIS-H_2SO_4 and 1 mM EDTA.

Claims

1. A method for renaturation of proteins comprising adding to a solution of denatured, chemically modified or reduced proteins a refolding buffer containing a primary, secondary or tertiary amine.

2. The method of claim 1 characterised in that the amine has the formula



wherein R_1 and R_2 can be any combination of the ligands H, O=C-NH_2, $(CH_2)_4$-NH_2, $(CH_2)_3$-COOH, $(CH_2)_2$-CHOH-CH_3, CH_2-CH_2-OH, CH_2-CH_3, CH_3, NH_2

and R_3 can be C(NH_2)=NH, C(CH_2OH)$_3$, CH_2-CH_2-OH or H

3. The method of any one of claims 1 or 2 wherein the buffer further contains a solubility enhancer.

4. The method of claims 3 wherein solubility enhancer is an ion.

5. The method of claims 3 wherein solubility enhancer is chloride or sulfate.

6. The method of claims 1 to 5 wherein the protein is an interleukin

7. The method of claims 1 to 5 wherein the protein is interleukin 4

8. The method of claims 1 to 5 wherein the protein is a mutein of interleukin 4.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/12607

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/54 C07K14/81 C07K1/113

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 01 87925 A (COX GEORGE N ;BOLDER BIOTECHNOLOGY INC (US); DOHERTY DANIEL H (US)) 22 November 2001 (2001-11-22) * claims 1,17,22,24,27; examples 1-3,5,8,12,14,19,22,23 *	1-8
X	WO 89 01046 A (SCHERING BIOTECH CORP) 9 February 1989 (1989-02-09) example 2	1-8

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
7 March 2003	20/03/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fausti, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

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